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Molecular epidemiology of penicillin-resistant *Streptococcus pneumoniae* in a university hospital, Ankara, Turkey

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ABSTRACT

Penicillin-resistant *Streptococcus pneumoniae* isolates (n = 76) from clinical samples of patients admitted to Hacettepe University Hospital between January 1997 and December 2001 were included in the study. MICs of penicillin G, erythromycin A, clindamycin, cefaclor, cefotaxime, vancomycin, chloramphenicol, tetracycline, ciprofloxacin and rifampicin were determined by agar dilution. The isolates were serogrouped on the basis of the Neufeld Quellung reaction and were typed by BOX-PCR. Genetic polymorphism of the penicillin resistance genes *pbp2b* and *pbp2x* was investigated by restriction fragment length polymorphism (RFLP) analysis. Of the 76 isolates tested, 64 (84.2%) showed intermediate resistance to penicillin, while 12 (15.8%) were resistant to higher levels of penicillin (MIC ≥ 2 mg/L). The resistance patterns of the isolates revealed six different resistance profiles. There were 22 different serotypes, with *c.* 55% of the isolates belonging to serotypes 23B, 19A, 19F, 14, 6 A and 9V. Five distinct patterns for *pbp2b* and 12 distinct patterns for *pbp2x* were obtained by RFLP analysis of penicillin-binding protein genes. The combination of these patterns allowed isolates to be classified into 22 fingerprint subgroups. BOX-PCR analysis showed that the isolates fell into 14 distinct BOX genotypes, with 33 subtypes. Serotype 9V isolates with *pbp* genotype 2-6 and BOX-PCR type 4, 4.1 or 4.2 were related to the pandemic clone Spain^{9V}-3. No relatedness to other international clones was detected among the other study strains, but genetic relatedness was observed among some of the serotype 19A and 23B isolates. Overall, the results demonstrated that most of the penicillin-resistant pneumococcal isolates in Turkey, other than those belonging to serotypes 9V, 19A and 23B, were derived from several independent clones, possibly resulting from multiple importation of strains originating from outside the country. Differences in *pbp* patterns, serotypes and resistance profiles among isolates that showed similar BOX-PCR patterns supported the hypothesis that horizontal transfer of capsular genes, *pbp* genes and other genetic determinants between *S. pneumoniae* and viridans group streptococci may have occurred.

Keywords BOX-PCR, multidrug resistance, *pbp*, penicillin resistance, serotyping, *Streptococcus pneumoniae*

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INTRODUCTION

Streptococcus pneumoniae remains an important human pathogen associated with significant morbidity and mortality [1, 2]. The prevalence of penicillin resistance among pneumococci is increasing alarmingly worldwide, and international spread of a restricted number of multiresistant pneumococcal clones has contributed significantly to this increase [2–4].

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The standard method of typing pneumococci is serotyping, and this has been used to follow the spread of penicillin-resistant *S. pneumoniae*. The value of this approach is limited, since many resistant isolates fall into a small number of serotypes [2]. Alternative approaches such as BOX-PCR (i.e., PCR involving pneumococcal repetitive sequences), pulsed-field gel electrophoresis, restriction fragment length polymorphism analysis of penicillin-binding protein (PBP) genes and multilocus sequence typing have been shown to be sensitive methods [4–7]. PBP genes of resistant pneumococci show a mosaic structure, and the different mosaic PBP genes can be used for

epidemiological purposes by analysing the patterns of PBP fingerprints [6]. It has been shown that the BOX-PCR assay, which targets a dispersed repetitive motif in the genome of *S. pneumoniae*, has equal or even greater resolving power than any of the other more laborious, complex and expensive DNA typing strategies [5].

In Turkey, the prevalence of penicillin-resistant pneumococci (MIC ≥ 0.1 mg/L) varies over the range 32–43.5%, with high-level penicillin-resistant isolates occurring at a frequency of 3–7% in certain centres [8–10]. This paper describes a molecular epidemiological study that was undertaken to identify the reasons for the high prevalence of penicillin-resistant pneumococci in our centre.

MATERIALS AND METHODS

Pneumococcal strains

In total, 76 penicillin-resistant *S. pneumoniae* isolates were chosen for molecular investigation from 179 *S. pneumoniae* strains isolated from clinical samples of patients admitted to Hacettepe University Hospital, Ankara during the period January 1997 to December 2001. A single pneumococcal isolate/patient/infection was analysed. *S. pneumoniae* species identification was on the basis of optochin susceptibility and bile solubility as described previously [8]. The pneumococci were collected from sputum (57.9%), bronchoalveolar lavage (14.6%), throat swab (10.6%), blood (3.9%), eye swab (3.9%), pus (2.6%), cerebrospinal fluid (2.6%), trans-tracheal aspirate (2.6%) and pleural fluid (1.3%). Isolates denoted with the capital letters B and P were from children, and those denoted with C were from adult patients.

Susceptibility testing

MICs were determined by the agar dilution method [8]. Breakpoints for intermediate and high-level penicillin resistance were according to National Committee for Clinical Laboratory Standards guidelines [11]. The antimicrobial agents tested were penicillin G, erythromycin A, clindamycin, cefaclor, cefotaxime, vancomycin, chloramphenicol, tetracycline, ciprofloxacin and rifampicin. *S. pneumoniae* strain ATCC 49619 was included in each batch of isolates tested as a quality control.

Macrolide–lincosamide–streptogramin$_B$ (MLS_B) resistance phenotypes were determined by a double-disk diffusion technique with erythromycin and clindamycin disks according to National Committee for Clinical Laboratory Standards guidelines [11]. The MLS_B phenotype was defined as resistance to erythromycin and clindamycin, while an isolate with the M phenotype was resistant to erythromycin alone.

Serotyping

Pneumococci were serotyped on the basis of the Neufeld Quellung reaction at the Centers for Disease Control (Atlanta, GA, USA).

DNA isolation

S. pneumoniae isolates were cultivated on sheep blood (5% v/v) agar in 5–10% CO_2 at 37°C overnight. Colonies were then emulsified in 100 μL TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5), and DNA was extracted by boiling [12] with an additional freeze–thaw step. DNA extracts were kept at − 20°C until used for PCR amplification.

BOX-PCR

Typing of pneumococci by BOX-PCR was performed as described by Van Belkum *et al.* [5]. Briefly, pneumococcal DNA was amplified by PCR with primer BOX-A. Amplified products were separated on an agarose 1.5% w/v gel. BOX-PCR patterns showing a single band difference were defined as non-identical types (i.e., 1–12). Identical banding patterns varying in the intensity of one or more bands were defined as subtypes (i.e., 1, 1.1, 1.2, etc.).

PBP genotyping

Genetic polymorphism of the penicillin resistance genes *pbp2b* and *pbp2x* was investigated by restriction fragment length polymorphism analysis as described by Coffey *et al.* [6]. Following PCR amplification of the PBP genes, the amplification products were digested with restriction endonuclease *Hin*fI at 37°C for 20 h. Digested DNA was separated by electrophoresis in agarose 1.8% w/v gels. The different PBP genotypes were identified by a number code.

BOX-PCR and *pbp2b* and *pbp2x* patterns of the major international antimicrobial resistant clones of *S. pneumoniae*, as defined by the Pneumococcal Molecular Epidemiology Network, were also determined by the same methods to compare the genotypes and to determine any possible clonal relationship between the Turkish isolates and these international clones. The 16 standard pneumococcal clones and their ATCC accession numbers were as follows: $Spain^{23F}$-1 (ATCC 700669), $Spain^{6B}$-2 (ATCC 700670), $Spain^{9V}$-3 (ATCC 700671), $Tennessee^{23F}$-4 (ATCC 51916), $Spain^{14}$-5 (ATCC 700902), $Hungary^{19A}$-6 (ATCC 700673), $South\ Africa^{19A}$-7 (ATCC 700674), $South\ Africa^{6B}$-8 (ATCC 700675), $England^{14}$-9 (ATCC 700676), CSR^{14}-10 (ATCC 700677), CSR^{19A}-11 (ATCC 700678), $Finland^{6B}$-12 (ATCC 700903), $South\ Africa^{19A}$-13 (ATCC 700904), $Taiwan^{19F}$-14 (ATCC 700905), $Taiwan^{23F}$-15 (ATCC 700906) and $Poland^{23F}$-16.

Computer analysis of BOX profiles

Genetic relatedness of the 76 study isolates and the 16 standard clones was determined by SPSS v.10.0 software (SPSS Inc., Chicago, IL, USA). Between-group linkage was used as a cluster method and squared Euclidean distance as a measure.

RESULTS

In total, 76 penicillin-non-susceptible pneumococcal strains were analysed by resistance profiles, serotyping, *pbp2b* and *pbp2x* genotyping, and BOX-PCR. Of these, 64 (84.2%) showed intermediate resistance to penicillin, while 12 (15.8%) were resistant to higher levels of penicillin (MICs

≥2 mg/L). Resistance to cefaclor, cefotaxime, erythromycin, clindamycin and chloramphenicol was significantly higher in penicillin-resistant isolates than in penicillin-intermediate isolates (Table 1). One-third of penicillin-resistant isolates were also resistant to cefotaxime. The overall resistance to erythromycin was 23.6% and that to clindamycin was 21%. Phenotyping of erythromycin-resistant isolates (n = 18) revealed that 11 (61.1%) had the $cMLS_B$ phenotype, five (27.8%) had the $iMLS_B$ phenotype, and two (11.1%) had the M phenotype. About 40% of the penicillin-resistant isolates were multiresistant, i.e., resistant to two or more different groups of antibiotics. Six different resistance profiles, ranging from resistance to β-lactams only to multidrug resistance against β-lactams, erythromycin, tetracycline and chloramphenicol, were observed. None of the isolates was resistant to vancomycin. One isolate was resistant to rifampicin and one to ciprofloxacin.

Serotyping revealed 20 different serotypes, namely, serotypes 23B (10 isolates), 19A (8), 19F (8), 14 (7), 9V (6), 6A (5), 23F (4), 9A (2), 4 (2), 11A (2), 15B (2), 1 (2), 3 (1) 6B (1), 7 (1), 9N (1), 16F (1), 10 (1), 31 (1), and 33A (1), in order of decreasing frequency. About 55% of the isolates belonged to serotypes 23B, 19A, 19F, 14, 9V and 6A. Ten (11.6%) isolates could not be serotyped, although proven to be *S. pneumoniae* by bile solubility. One of these non-typeable isolates was from a cerebrospinal fluid sample, and one from a pleural fluid sample, while the others were from the respiratory tract.

Table 1. Antibiotic susceptibilities of 76 *S. pneumoniae* isolates (64 isolates showing intermediate (I) resistance to penicillin, and 12 strains showing resistance (R) to penicillin) subdivided according to their level of penicillin resistance

Antibiotic	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	% Resistance
Penicillin				
I	0.125–1	0.25	1	0
R	2–8	2	4	100.0
Cefaclor				
I	0.125–4	1	4	23.4
R	4–> 32	8	> 32	100.0
Cefotaxime				
I	≤ 0.06–0.5	0.125	0.25	0
R	0.5–2	0.5	4	33.3
Erythromycin				
I	≤ 0.06–> 32	0.125	8	23.4
R	≤ 0.06–16	0.125	4	25.0
Clindamycin				
I	≤ 0.06–> 32	0.125	2	20.3
R	≤ 0.06–> 32	0.125	> 32	25.0
Chloramphenicol				
I	0.25–8	2	4	3.1
R	1–16	2	8	16.7
Tetracycline				
I	≤ 0.06–> 32	0.25	16	28.1
R	0.25–16	0.5	8	16.7
Vancomycin				
I	≤ 0.06–0.5	0.25	0.5	0
R	0.25–0.5	0.5	0.5	0
Ciprofloxacin				
I	0.5–> 8	1	2	1.5
R	0.5–1	1	1	0
Rifampicin				
I	≤ 0.03–4	0.03	0.03	1.5
R	≤ 0.03–0.125	0.03	0.06	0

Genetic heterogeneity in the penicillin resistance genes was investigated by PBP genotyping of the *pbp2b* and *pbp2x* genes. Five distinct patterns were obtained for *pbp2b* and 12 distinct patterns for *pbp2x*. By combining these patterns, 22 fingerprint subgroups (namely 1-1 to 1-12, 2-1, 2-3, 2-6, 2-8, 3-1, 3-5, 4-1, 4-3, 4-5 and 5-11) could be defined. When the MIC values were evaluated in relation to the PBP variations, it was seen that there was more variation in *pbp2x* genes than in *pbp2b* genes, which seemed to be associated with the low-level penicillin resistance expressed in those isolates.

BOX-PCR analysis divided the isolates into 14 distinct BOX genotypes, with 33 subtypes. When the BOX patterns were evaluated together with serotypes and PBP types, two isolates (B8 and B78) were identical to the $Spain^{9V}$-3 clone, while four other isolates (C80, B81, C78 and B55) were related closely to the same clone. No relatedness to the international clones was detected among the other isolates in the study.

Serotype 23B isolates B64, B66, B60 and B62 were identical to each other when BOX-PCR types, PBP types and serotypes were taken into consideration.

Isolates P11, P37, B41, B80, B85, B71 and B73 were apparently identical by BOX-PCR patterns, but only B71 and B73 were identical both by serotype (19A) and PBP type, while P11 had the same PBP pattern, but belonged to serotype 6A, indicating possible horizontal transfer of capsular genes.

There was also a group of isolates with identical BOX patterns, but with a high degree of heterogeneity in terms of PBP types and serotypes (i.e., isolates C40, C79, B11, P66, C35, P20, P63, P4, P8, B74, B77 and B53). Some of these isolates seemed to be related genetically, since they had the same serotype and *pbp2b* and/or *pbp2x* types.

DISCUSSION

Resistance to penicillin is increasing rapidly worldwide among strains of *S. pneumoniae*. Molecular analysis has shown that a specific strain of penicillin-resistant pneumococcus may spread from country to country, and even between continents. The prevalence of pneumococci with penicillin MICs > 0.1 mg/L is 32–44% in Turkey [8–10]. Although this is not a nationwide study on the prevalence and molecular epidemiology of penicillin-resistant pneumococci in Turkey, it reflects the overall picture, since the isolates in the study were from patients admitted to this centrally located reference hospital from different parts of the country.

The presence of multiresistance in *c.* 40% of the penicillin-resistant isolates has serious therapeutic implications. β-Lactams and macrolides are obtained frequently 'over-the-counter' in Turkey, favouring rapid selection of pneumococci with reduced susceptibility. Phenotypic characterisation of the macrolide-resistant pneumococci in this study revealed that most expressed the $cMLS_B$ phenotype, a result consistent with findings in many other European countries [13]. Acquisition and spread of β-lactam resistance in pneumococci are complex processes. Close contact among children and frequent antibiotic treatment favour the selection and spread of resistant pneumococci. Most of the *S. pneumoniae* isolates tested in this study were from children who were receiving antibiotics because of frequent respiratory tract infections.

Some genetic (PBP pattern) and phenotypic (serotype and resistance profile) differences were observed among isolates with similar BOX-PCR patterns, which might be attributed to the horizontal transfer of capsular genes, PBP genes and other genetic determinants between these isolates and other pneumococcal strains or viridans streptococci. Serotyping revealed that 55% of the isolates belonged to serotypes 23B, 19A, 19F, 14, 9V and 6A, which are covered in the seven-valent conjugate vaccine. These data correspond largely to earlier findings in other European countries, the USA and the Far East, in which these serogroups also contributed significantly to the prevalence of penicillin-resistant pneumococci [14–20]. About 11% of the isolates could not be serotyped with the available typing system. Since about 82% of these isolates were from the respiratory tract, these may be contaminants rather than true pathogens.

The discriminatory power of serotyping is considered to be poor, since different genotypes may express the same phenotypic characteristics and, as *S. pneumoniae* is a naturally transformable species, frequent exchange of capsular genes may occur [6, 21, 22]. This was confirmed in the present study by molecular typing, which demonstrated that isolates from most serotypes were genetically heterogeneous, and that many clones were detected during the study period. However, penicillin-resistant pneumococci of serotypes 23B and 9V appeared to be more clonal. Further evaluation of these isolates revealed that the serotype 9V isolates with PBP genotype 2-6 and BOX-PCR type 4, 4.1 or 4.2 were related to the pandemic clone $Spain^{9V}$-3 [4], providing further evidence that antibiotic-resistant isolates expand clonally because of an evolutionary advantage. Thus, the $Spain^{9V}$-3 clone was identified first in Spain [6] and France [23], and then subsequently in the UK, Italy, Germany, the USA and the Far East [6, 17, 18, 24, 25]. Similarly, worldwide spread of the $Spain^{23F}$-1 clone from Spain to the USA was reported by Munoz *et al.* [3], and then subsequently to various regions of the world, including the UK, Bulgaria, Germany, France, South Africa and Portugal [6, 14, 18, 23, 26, 27]. This is the first report from Turkey on the possible presence of the $Spain^{9V}$-3 pandemic clone in our country.

Overall, these results suggest that most penicillin-resistant pneumococcal isolates in Turkey, other than serotypes 9V and 23B, were derived from several independent clones. It is possible that the diversity of resistant genotypes detected in this study may be associated with multiple importation of strains originating from outside the country, particularly as Turkey is one of the main transit countries between East and West. Although some of the serogroup 23 and 9 isolates may have originated from a common ancestor, multiplication of these possible clones does not seem to be the only process explaining penicillin-resistant pneumococcal spread in our region. However, in such a densely populated country, it is always possible that particular multidrug-resistant pneumococcal clones may become successful and disseminate widely throughout the country.

Several isolates showed diversity among PBP genes, indicating independent mutation of PBP

genes, or horizontal transfer of PBP genes between strains. The overall data were consistent with the likelihood that all or most of the penicillin-resistant isolates described here carried mosaic *pbp2b* and *pbp2x* gene sequences that originated from past recombination events with other streptococcal species. It is well known that strains with similar BOX profiles can be of different serotype, PBP type and even pulsed-field gel electrophoresis type. The heterogeneity of PBP types in the same BOX clusters obtained in this study could be attributed to this.

The increasing prevalence of penicillin and multidrug-resistant pneumococci in Turkey emphasises the importance of judicious use of antibiotics and vaccination to prevent serious pneumococcal infections in people at greater risk for pneumococcal disease.

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REFERENCES

1. Appelbaum PC. Resistance among *Streptococcus pneumoniae*: implications for drug selection. *Clin Infect Dis* 2002; **34**: 1613–1620.
2. Adam D. Global antibiotic resistance in *Streptococcus pneumoniae*. *J Antimicrob Chemother* 2002; **50**(suppl): 1–5.
3. Munoz R, Coffey TR, Daniels M *et al*. Intercontinental spread of a multiresistant clone of serotype 23F *Streptococcus pneumoniae*. *J Infect Dis* 1991; **164**: 302–306.
4. McGee L, McDougal L, Zhou J *et al*. Nomenclature of major antimicrobial-resistant clones of *Streptococcus pneumoniae* defined by the Pneumococcal Molecular Epidemiology Network. *J Clin Microbiol* 2001; **39**: 2565–2571.
5. Van Belkum A, Sluijter M, De Groot R, Verbrugh H, Hermans PWM. Novel BOX repeat PCR for high resolution typing of *Streptococcus pneumoniae* strains. *J Clin Microbiol* 1996; **34**: 1176–1179.
6. Coffey TJ, Dowson CG, Daniels M *et al*. Horizontal transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of *Streptococcus pneumoniae*. *Mol Microbiol* 1991; **5**: 2555–2560.
7. Enright MC, Spratt BG. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. *Microbiology* 1998; **144**: 3049–3060.
8. Şener B, Günalp A. Trends in antimicrobial resistance of *Streptococcus pneumoniae* in children in a Turkish hospital. *J Antimicrob Chemother* 1998; **42**: 381–384.
9. Gür D, Güçiz B, Hascelik G *et al*. *Streptococcus pneumoniae* penicillin resistance in Turkey. *J Chemother* 2001; **13**: 541–545.
10. Yenişehirli G, Şener B. Antibiotic resistance and serotype distribution of *Streptococcus pneumoniae* strains isolated in Hacettepe University Hospital. *Mikrobiyol Bult* 2003; **37**: 1–11.
11. National Committee for Clinical Laboratory Standards. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*, 5th edn. Approved standard M7-A5. Wayne, PA: NCCLS, 2000.
12. Sambrook J, Russel DW. *Molecular cloning, a laboratory manual*. New York, NY: Cold Spring Harbor Laboratory Press, 2001; 1.43–1.50.
13. Schmitz FJ, Perdikouli M, Beeck A, Verhoef J, Fluit AC. Molecular surveillance of macrolide, tetracycline and quinolone resistance mechanisms in 1191 clinical European *Streptococcus pneumoniae* isolates. *Int J Antimicrob Agents* 2001; **18**: 433–436.
14. Setchanova L, Tomasz A. Molecular characterization of penicillin-resistant *Streptococcus pneumoniae* isolates from Bulgaria. *J Clin Microbiol* 1999; **37**: 638–648.
15. Overweg K, Hermans PWM, Trzcinski K, Sluijter M, De Groot R, Hryniewicz W. Multidrug-resistant *Streptococcus pneumoniae* in Poland: identification of emerging clones. *J Clin Microbiol* 1999; **37**: 1739–1745.
16. Linares J, Garau J, Dominguez C, Perez JL. Antibiotic resistance and serotypes of *Streptococcus pneumoniae* from patients with community acquired pneumococcal disease. *Antimicrob Agents Chemother* 1983; **23**: 545–547.
17. Marchese A, Ramirez M, Schito GC, Tomasz A. Molecular epidemiology of penicillin-resistant *Streptococcus pneumoniae* isolates recovered in Italy from 1993 to 1996. *J Clin Microbiol* 1993; **36**: 2944–2949.
18. Reichman P, Varon E, Günther E *et al*. Penicillin-resistant *Streptococcus pneumoniae* in Germany: genetic relationship to clones from other European countries. *J Med Microbiol* 1995; **43**: 377–385.
19. McGee L, Klugman KP, Tomasz A. Serotypes and clones of antibiotic-resistant pneumococci. In: Tomasz A, ed. *Streptococcus pneumoniae: molecular biology and mechanisms of disease*. New York, NY: Mary Ann Liebert Inc., 2000; 375–379.
20. Ip M, Lyon DJ, Yung RWH, Chan C, Cheng AFB. Evidence of clonal dissemination of multidrug-resistant *Streptococcus pneumoniae* in Hong Kong. *J Clin Microbiol* 1999; **37**: 2834–2839.
21. Hermans PWM, Overweg K, Sluijter M, de Groot R. Penicillin-resistant *Streptococcus pneumoniae*: an international molecular epidemiological study. In: Tomasz A, ed. *Streptococcus pneumoniae: molecular biology and mechanisms of disease*. New York, NY: Mary Ann Liebert Inc., 2000; 457–466.
22. Nesin M, Ramirez M, Tomasz A. Capsular transformation of a multi-drug resistant *Streptococcus pneumoniae* in vivo. *J Infect Dis* 1998; **177**: 707–713.
23. Lefevre JC, Bertrand MA, Faucon G. Molecular analysis by pulsed-field gel electrophoresis of penicillin-resistant *Streptococcus pneumoniae* from Toulouse, France. *Eur J Clin Microbiol Infect Dis* 1995; **14**: 491–497.

24. Corso A, Severina EP, Petruk VF, Mauriz YR, Tomasz A. Molecular characterization of penicillin-resistant *Streptococcus pneumoniae* isolates causing respiratory disease in the United States. *Microb Drug Resist* 1998; **4**: 325–337.
25. Dejsirilert S, Overweg K, Sluijter M *et al*. Nasopharyngeal carriage of penicillin-resistant *Streptococcus pneumoniae* among children with acute respiratory tract infections in Thailand: a molecular epidemiological survey. *J Clin Microbiol* 1999; **37**: 1832–1838.
26. Klugman KP, Coffey TJ, Smith A, Wasas A, Myers M, Spratt BG. Cluster of erythromycin resistant variant of the Spanish multiply resistant 23F clone of *Streptococcus pneumoniae* in South Africa. *Eur J Clin Microbiol Infect Dis* 1994; **13**: 171–174.
27. Vaz Pato MV, de Carvalho CB, Tomasz A, the Multicenter Study Group. Antibiotic susceptibility of *Streptococcus pneumoniae* isolates in Portugal. A multicenter study between 1989 and 1993. *Microb Drug Resist* 1995; **1**: 59–69.